Levels of expression of hRPB11, a core subassembly subunit of human RNA polymerase II, affect doxorubicin sensitivity and cellular differentiation

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Received 11 March 1998

Abstract We have previously shown that the human RNA polymerase II subunit 11 (hRPB11) is among the proteins specifically downregulated upon Doxorubicin (Dox) treatment of human cancer cell lines, and that Dox resistant clones derived upon drug selection express about 20% of the protein present in the original parental cell line. Given the prominent role that this subunit appears to have in eukaryotic cells, and the fact that its deletion causes lethality in yeast, we wanted to test the effect of the reintroduction of parental cell line levels of this subunit in Dox resistant colon cancer cells (LoVoDX). Stable transfectants of LoVoDX expressing parental (LoVoH) levels of hRPB11 showed a reduced sensitivity to the drug without changing the response of these cells to other chemotherapeutic agents, confirming a specific inverse correlation between cellular Dox sensitivity anti-hRPB11 levels of expression. In addition we show here that the levels of expression of this same RNA polymerase II subunit directly affect cellular differentiation, reducing the rate of cell proliferation, clonogenicity and increasing the expression of E-cadherin, a marker of epithelial cell differentiation. As expected from cells with these characteristics, upon in vivo administration of these clones in nude mice, we detected a significant reduction in the size and time of appearance of the primary tumors and overall metastatic capability. Finally, the role played by hRPB11 in regulating the transcription of specific genes is underlined by transient transfection experiments that show transactivation of the E-cadherin promoter by this protein.

Key words: Doxorubicin; Drug toxicity; RNA polymerase II; Cell differentiation

1. Introduction

One of the major obstacles encountered during cancer chemotherapy is the emergence of cellular drug resistance with consequent selection of more aggressive tumor phenotypes. It has been shown that the appearance of this phenomenon is associated with altered expression of proto-oncogenes, cell cycle and apoptosis regulating genes [1–5]. Doxorubicin (Dox) is a widely used antineoplastic drug with a broad spectrum of chemotherapeutic activity on several malignancies [6], but its use is often hampered by the surging of multidrug resistance [7,8] and its cumulative dose-dependent cardiac toxicity, which leads to irreversible degenerative cardiomyopathy [9].

We [10] and others [11,12] have shown that Dox exerts specific functions on the transcription machinery without affecting housekeeping genes. In particular, regarding the myocardium, it has been demonstrated that Dox negatively affects the transcription of heart-specific genes [11–13]. Among the causes of this phenomenon the induction of a dominant negative regulator of transcription has been observed [14]. These latter findings have been postulated to play a pathogenic role in the degenerative cardiomyopathy associated with Dox administration.

Interestingly, among the genes specifically affected by Dox administration, we have previously identified and cloned hRPB11, a core subassembly subunit of RNA polymerase II (RNA pol II) (for recent nomenclature see [15]) as the enzyme involved in the synthesis of mRNA [16–18] and have shown a dramatic correlation between Dox treatment and downmodulation of this ubiquitous protein in different cell lines [10]. Therefore we sought to investigate the possible correlation between levels of expression of this subunit and Dox cellular sensitivity and ultimately how Dox treatment could influence the progression toward a more aggressive neoplastic phenotype. We report here that reintroduction of parental cell line (LoVoH) levels of hRPB11 in Dox resistant colon cancer cell (LoVoDX) dramatically increases the cell sensitivity to this chemotherapeutic agent without affecting the response to other antineoplastic drugs. In addition, cells stably transfected with hRPB11 show a profound modification of their phenotype accompanied by increased levels of expression of E-cadherin and a less aggressive behavior when administered in vivo. Finally the effects of hRPB11 in promoting specific gene transcription are made tangible in transient transfection experiments, showing that the E-cadherin promoter linked to a reporter gene is transactivated when hRPB11 is cotransfected in the same cell. Taken together these findings suggest that a direct correlation exists between hRPB11 levels of expression and Dox sensitivity and that the same may also play a major role in cell differentiation.

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Abbreviations: Dox, doxorubicin; pol II, polymerase II; hRPB11, human RNA polymerase II subunit 11; hRPB3, human RNA polymerase II subunit 3

PII S0014-5793(98)00432-3

2. Materials and methods

2.1. Cells analysis of drug sensitivity and in vitro proliferation

Human colon carcinoma LoVoH and LoVoDX (Dox resistant cells) were kindly provided by Dr. M.P. Colombo (Istituto Nazionale Tumori, Milan, Italy). The establishment and characteristics of these cells have already been described [13]. LoVoDX cells were maintained by continuous exposure to 0.2 μM Dox (Adriblastina, Farmitalia Carlo Erba, Milan, Italy). Drug sensitivity of LoVoDX transfectants was evaluated by calculating the drug dose responsible for 50% inhibition of proliferation (IC_{50} value). Cells were plated in triplicate at the density of 2×10^5 cells/60 mm diameter tissue culture dishes. After 24 h of incubation in Ham's F12 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (Life Technologies, Gaithersburg, MD, USA), cells were treated with the appropriate drug concentrations. After 8 days, dishes were washed with PBS, cells were detached by trypsin treatment and counted using a Coulter counter model ZM (Coulter Electronics, Luton, UK). All cell lines were examined simultaneously and all experiments were performed at least three times. Proliferation curves were obtained by plating 1×10^5 cells into 60-mm diameter dishes. At daily intervals, cells were detached by trypsin treatment and counted using a Coulter counter model ZM. Doubling time was calculated from the exponential region of proliferation curves. Clonogenic ability was evaluated by plating 5×10^2 cells into 60-mm dishes, so that colonies would appear after 8–10 days. Cells were fixed and stained with 0.5% Crystal Violet solution containing 20% ethanol. Colonies consisting of >50 cells were counted using a light microscope. The clonogenic ability was calculated as percentage of colony numbers over plated cell number. All experiments were performed at least three times with three samples for each cell line.

2.2. Western blot analysis of hRPB11 and E-cadherin expression

Equal amounts of total cell lysates were separated on 12.5% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [20]. Proteins were transferred to nitrocellulose (Amersham, Bucks, UK) and blots were probed with a purified polyclonal anti-hRPB11 antibody [10]. For E-cadherin analysis a 6% gel was used and the blot was probed with a specific monoclonal antibody anti-E-cadherin (Transduction Laboratories, Lexington, KY, USA). Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham) as described by the manufacturer. Band intensity from Western blot analysis was quantified using a GS-700 Imaging Densitomer (Bio-Rad) and normalized to the relative HSP7O protein levels detected with a specific monoclonal antibody (W27, Santa Cruz Biotech. Inc., Santa Cruz, CA, USA).

2.3. RT-PCR analysis of E-cadherin mRNAs

The analysis of E-cadherin mRNA expression was carried out as previously described [21]. PCR products were electrophoresed onto a 1.5% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized under UV light. The relative intensity of the bands was quantified by densitometric analysis (GS-Imaging Densitometer, Bio-Rad, Hercules, CA, USA) and normalized to a co-amplified aldolase cDNA fragment as previously reported [22]. At least 3 RT-PCR experiments were performed using three different RNA preparations.

2.4. DNA transfections and analysis of E-cadherin promoter activity

LoVoDX cells overexpressing hRPB11 were obtained by stable transfection using the expression vector pBK-hRPB11, made by ligating the entire hRPB11 cDNA into pBK-CMV (Stratagene, La Jolla, CA, USA). Cells were transfected using the calcium phosphate procedure [23] and selected in 1 mg/ml G-418 for 10 days, and then maintained at 0.6 mg/ml. Individual lines were derived by isolating single primary colonies. LoVoDX cells were also transfected with the empty vector pBK-CMV and the G418 resistant clones obtained were used as control in all of the experiments performed. For the analysis of the human E-cadherin promoter activity, transient transfection experiments were performed using the luciferase reporter gene construct pGL2 Ecad3/Luc containing E-cadherin 5′ flanking sequences of 1484 bp, kindly provided by Dr. E.C. Fearon [24]. The pGL2-control luciferase reporter vector was obtained from Promega (Promega, Madison, WI, USA). The pCMVβ plasmid, containing a functional LacZ gene expressed under the control of the human cytomegalovirus immediate early promoter, was obtained from Clontech (Clontech, Palo Alto, CA, USA). LoVoDX cells growing at roughly 70% confluence were transfected in 60-mm plates with 3 μg of the pGL2 plasmids, 3 μg of pBK-CMV or pBK-hRPB11, and 0.5 μg of pCMVβ plasmid, using the calcium phosphate procedure [23]. Cell extracts were prepared 48 h post transfection using reporter lysis buffer (Promega). One tenth of the lysate was used for the luciferase and β-galactosidase assays, carried out as recommended by the manufacturers, Promega and Tropix (Tropix, Bedford, MA, USA), respectively. Luciferase and β-galactosidase activities were measured with a luminometer (model MLX, Dynex Technologies, Chantilly, VA, USA). All transfections were repeated at least three times.

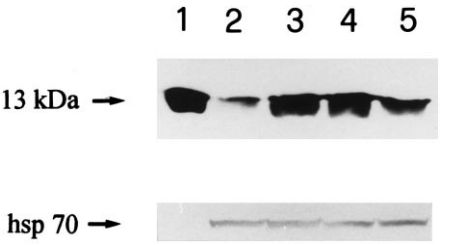

Fig. 1. Transfection of LoVoDX cells with pBK-hRPB11 restores LoVoH levels of expression of the protein. Top: Detection of hRPB11 protein in LoVoDX stable transfectants. Protein lysates were separated by SDS-PAGE and hRPB11 was detected by immunoblotting with a specific polyclonal antibody [10]. Lane 1: recombinant fusion protein used as hRPB11 positive control; lane 2: LoVoDX cells stably transfected with empty vector, clone pBK-3; lanes 3,4: hRPB11 stable transfectants clone alpha and gamma, respectively; lane 5: LoVoH cells. Bottom: Results obtained on the same blot with an anti-HSP70 monoclonal antibody.

2.5. Animals and in vivo experiments

Male CD-1 nude (nu/nu) mice (6–8 weeks old and 22–24 g in body weight) were purchased from Charles River Laboratories, Calco, Italy. Animals were kept under pathogen-free conditions in a laminar air-flow cabinet and fed with acidified, autoclaved water and γ-irradiated commercial diet ad libitum. All manipulations were performed under sterile conditions in a laminar air flow hood. All procedures involving animals were in accordance to institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/109, OJL 358, December 1, 1987, and the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, NIH Publ. No. 85-23, 1985). LoVoDX and LoVoDX-hRPB11 expressing cells in exponential phase of growth were harvested, washed once with medium and resuspended in cold medium without FCS. 5×10^6 viable cells, as evaluated by the trypan blue exclusion test, were concentrated in 0.2 ml of serum-free medium and injected into the hind leg muscles of the mice. Each experimental group included ten mice. Neoplastic development was monitored and tumor weights were calculated from caliper measurements according to the formula: tumor weight (mg) = length (mm) × width^2 (mm)/2 [25]. To evaluate the metastatic ability, tumor-bearing mice were sacrificed 120 days after tumor cell injection. Their lungs were removed

Table 1
Doxorubicin sensitivity of transfected LoVoDX-hRPB11 cells

Cells	IC_{50} (μM)	Fold sensitivity
LoVoDX	4.10	
pBK-1	4.00	0.97
pBK-2	4.20	1.02
pBK-3	4.20	1.02
alpha	1.50	2.73
beta	1.00	4.10
gamma	0.75	5.46

Experiments were performed as described in Section 2. IC_{50} values correspond to the drug responsible for 50% of cell proliferation inhibition. Fold sensitivity corresponds to the ratio of IC_{50} parental cells. The data shown represent the mean of three independent experiments performed in triplicate.

Table 2
Drug sensitivity analysis of LoVoDX-hRPB11 transfectants

Drug	IC_{50} (μM)						
	LoVoDX	pBK-1	pBK-2	pBK-3	alpha	beta	gamma
Doxorubicin	4.10	4.00	4.20	4.20	1.50	1.00	0.75
Cisplatin	400	430	400	380	425	375	425
Etoposide	2.80	2.80	2.70	2.70	3.00	2.80	3.00
Actinomycin D	0.05	0.05	0.06	0.05	0.04	0.04	0.05
Vinblastine	0.05	0.05	0.04	0.04	0.06	0.05	0.05

Experiments were performed as described in Section 2. IC_{50} values correspond to the drug dose responsible for 50% inhibition of cell proliferation. The data shown represent the mean of three independent experiments performed in triplicate.

and fixed in Bouin's solution to distinguish tumor nodules from lung tissue, and the number of metastases was determined with the aid of a dissecting microscope.

2.6. Statistical analysis

Statistical analysis was performed by the Mann-Whitney U test for statistical significance. Differences were considered significant at P values < 0.05 (two sided).

3. Results

3.1. Re-expression in LoVoDX cells of hRPB11 levels

We have previously shown that the level of hRPB11 protein is decreased in LoVoDX resistant cells by about 80% when compared to the parental cell line LoVoH [10]. To test our hypothesis that this downregulation could play an important role in Dox sensitivity, LoVoDX cells were transfected with either pBK-hRPB11 or the empty vector pBK-CMV, and stable transfectants were generated. Three G418-resistant clones derived from either PBK-hRPB11 or empty vector transfections were isolated and expanded for further analysis. In order to confirm hRPB11 protein overexpression in transfected LoVoDX cells, immunoblot analysis was performed using a purified anti-hRPB11 polyclonal antibody [10]. As shown in Fig. 1, hRPB11 transfected clones alpha and gamma expressed much higher levels of hRPB11 protein than the control transfected cells. In addition their level of expression was similar, quantitatively, to the one present in Dox sensitive LoVoH cells (Fig. 1, lane 5 and [10]). Clone beta expressed similar levels on the protein (not shown).

3.2. hRPB11 overexpression specifically affects cellular Dox sensitivity

To test if cellular sensitivity to Dox was correlated to the levels of hRPB11 expression, we evaluated the pattern of Dox sensitivity in hRPB11 transfectant and control clones. Table 1 shows that each stable transfected clone displayed an increased sensitivity to Dox, with an IC_{50} ranging from 0.75 to 1.50 μM, remarkably lower than that of control tranfectants pBK-1, pBK-2 and pBK-3, that essentially exhibited the same IC_{50} of parental LoVoDX cells (4.0 μM). These observations clearly indicate that overexpression of hRPB11 confers an increased sensitivity to Dox treatment. Since the decrease in sensitivity could be explained by effects on the MDR1 gene expression with resulting selective resistance to drugs that are transported by this membrane pump [4], we evaluated whether the transfection of hRPB11 affected also the sensitivity of hRPB11 transfectants to other antineoplastic drugs. Table 2 shows that in these experiments the IC_{50} values for all compounds tested did not indicate any significant difference between controls and hRPB11-transfected clones, demonstrating that hRPB11 expression induces a very selective effect on Dox sensitivity and thus suggesting that hRPB11 exerts its effect through a specific pathway not involving the MDR1 gene.

3.3. hRPB11 overexpression affects morphology of LoVoDX cell

LoVoDX cells display a peculiar morphology, reminiscent of less differentiated phenotype, in respect to Dox sensitive LoVoH cells [19]. Interestingly, when transfected with hRPB11 LoVoDX cell clones grew in monolayers, displaying cell clusters and epithelial-like sheets with a cellular polygonal shape (Fig. 2, panel B) remarkably different from the rounded-up shape of LoVoDX control cells (Fig. 2, panel A), showing that hRPB11 overexpression profoundly affects the cellular phenotype. Similar morphological profiles were obtained from each one of the hRPB11 transfectant clones (not shown). These features are characteristic of a more differentiated cell phenotype [26].

3.4. Growth inhibitory activity of hRPB11

These latter findings, underscoring additional roles for hRPB11, prompted us to analyze the growth characteristics of the hRPB11 transfectant clones. Table 3 shows that in hRPB11 transfectant clones we observed an increase of doubling time and a reduction of saturation density in comparison to control clones and LoVoDX cells. To confirm the growth inhibitory activity of hRPB11 we evaluated the clonogenic ability of transfected clones. While we detected a decrease of about 50% of clonogenic ability in hRPB11 transfected clones, we did not observe any difference between control transfectant clones and LoVoDX cells. In addition, hRPB11 transfectant clones also exhibited a reduction in their proliferative rate (not shown).

Table 3
Effects of hRPB11 transfection on cell growth

Cells	Doubling time (hours) ± S.D.	Saturation density (cells × 10^6) ± S.D.	Clonogenic ability (%)
LoVoDX	18 ± 3	3.3 ± 0.2	18.4
pBK-1	22 ± 2	3.1 ± 0.4	16.5
pBK-2	21 ± 1	3.3 ± 0.3	16.3
pBK-3	20 ± 2	3.4 ± 0.5	15.8
alpha	34 ± 5	2.1 ± 0.2	10.6
beta	27 ± 3	2.0 ± 0.2	9.5
gamma	29 ± 5	2.1 ± 0.3	9.6

Experiments were performed as described in Section 2. Clonogenic ability was evaluated as percentage of colony numbers over plated cell number. The data shown represent the mean of three independent experiments performed in triplicate.

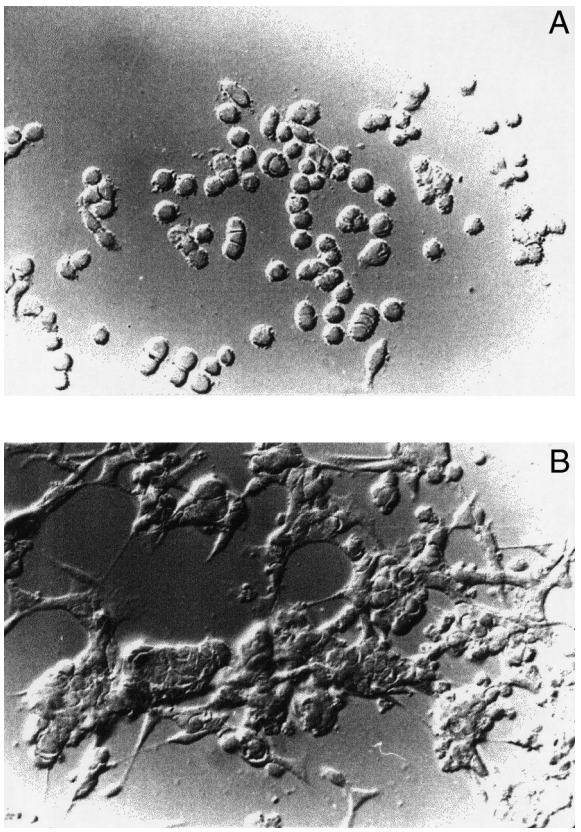

Fig. 2. hRPB11 transfectants display a more differentiated phenotype. A: Cell morphology of pBK-3 cells. B: Cell morphology of hRPB II transfectant cells, clone gamma. Compared to parental LoVoDX and empty vector transfected cells, hRPB11 transfectants displayed strong morphological modifications, forming clusters and epithelial like sheets.

3.5. Transfection of hRPB11 increases E-cadherin expression

The downmodulation or disappearance of several markers of differentiation characterizes tumor progression. Among these, E-cadherin [27] expression is found to be at a lower level in poorly differentiated colon carcinomas when compared to either normal colon epithelium or well-differentiated colon carcinomas [28]. Furthermore, several studies have suggested an inverse correlation between E-cadherin expression and tumor grade [28–30]. We analyzed the E-cadherin expression at both the mRNA and protein levels in LoVoDX-hRPB11 or control transfected cells. Table 4 shows that hRPB11 overexpressing clones, when compared to controls, displayed an increase of E-cadherin expression, confirming the observation that overexpression of hRPB11 results in the appearance of a more differentiated cellular phenotype.

Table 4
Analysis of E-cadherin expression in LoVoDX-hRPB11 transfectants

Cells	mRNA expression	Δ%	Protein expression	Δ%
LoVoDX	2.20	–	1.20	2.5
pBK-1	2.32	5	1.23	2.5
pBK-2	2.15	–	1.23	9
pBK-3	2.40	9	1.25	4.1
alpha	3.44	56	1.74	45
beta	3.24	47	1.78	48
gamma	3.76	70	1.60	33

RT-PCR and Western blot analysis were performed as described in Section 2. Data represent the OD ratio of mRNA or protein expression normalized to aldolase or HSP70, respectively. Δ% corresponds to the percentage of increased expression over parental cells. The data shown represent the mean of three independent experiments.

3.6. HRPB11 overexpression reduces tumorigenicity in nude mice

Since the overall data appeared to show that a more differentiated in vitro phenotype was the result of hRPB11 overexpression, we wanted to test the in vivo behavior of these transfectants. To this purpose, we inoculated exponentially growing cells of gamma and pBK-3 clones into nude mice and followed the progression of tumor formation. As shown in Table 5, tumor appearance was significantly delayed in animals inoculated with gamma with respect to control cells. Mice injected with 5×10^6 pBK-3 cells showed a palpable tumor with a median time of 29 days after cell inoculation, whereas 43 days ($P=0.032$) were necessary to make the same observation in the gamma cells injected mice. At day 50 after tumor cell inoculation, the average weight of pBK-3-derived tumors was 670 mg, whereas the tumor formed by the gamma clone cells was 263 mg ($P=0.008$). In addition an analysis of spontaneous lung metastases evaluated by sacrificing the animals at 120 days after tumor cell injection, revealed that gamma cell inoculated mice also showed a reduced number of lung metastates (Table 5, $P=0.03$). Thus, it appears that the more differentiated phenotype observed in vitro in hRPB11 overexpressing clones is paralleled in vivo by reduced tumor growth and metastatic ability.

3.7. hRPB11 transactivates E-cadherin promoter

It has recently been shown that among the mechanisms underlying the loss of E-cadherin expression in epithelial cancers, defects in transacting pathways play a major role [24]. Since an essential component of the basal transcription machinery. hRPB11 modulates E-cadherin levels of expression, we proceeded to investigate if hRPB11 could be a specific transacting factor for the E-cadherin gene promoter. To this purpose, we transiently transfected pBK-hRPB11 DNA together with E-cad3/Luc vector [24] into LoVoDX cells. The hRPB11 cDNA increased the E-cadherin promoter activity more than 8-fold over the basic reporter construct, when co-transfected with Ecad3/Luc (Fig. 3). These findings imply that hRPB11 has a direct role in regulating E-cadherin expression through transcriptional mechanisms.

4. Discussion

The data presented in this paper show a specific involvement of hRPB11 expression in Dox mediated cellular toxicity, the sensitivity to the drug being inversely correlated to the levels of hRPB11. Moreover, we show that levels of this protein, a core element of the transcription apparatus, deeply affect cell differentiation, changing cell proliferation, expression of peculiar markers, in vivo tumorigenicity and metastatic capability. Finally, as expected from a molecule involved in mRNA transcription, in the case of the E-cadherin promoter hRPB11 acts as a transacting factor.

We have previously shown that Dox specifically downregulated the levels of hRPB11 either as a consequence of the

Table 5
In vivo tumorigenicity and metastatic behavior of a LoVoDX-hRPB11 transfectant

Cells	Median latency of tumor appearance in days (range)	Tumor weights (mg) ± S.D.	Number of spontaneous metastases (range)
LoVoDX pBK-3	29 (27–41)	670 ± 228	50 (40–60)
LoVoDX gamma	43 (34–45)*	263 ± 43**	32 (20–37)

Male CD-1 nude mice were injected into the hind leg with 5×10^6 cells. Tumor weights were assessed at day 50 after cell inoculation, lung metastases at day 120. Each experimental group included 10 mice. $^*P<0.05$; $^{**}P<0.01$.

selection of Dox resistant clones or in the course of acute administration to several human cell lines [10]. We show in this report that the inverse is true, since restoration of hRPB11 levels detected in non-Dox treated cells (LoVoH) is responsible for a selective increase in cell sensitivity to this chemotherapeutic agent which does not involve the product of the MDR gene [7,8].

Dox, in addition to displaying specific systemic toxic side effects [31–34], selectively impairs the function of several genes. Indeed, it downmodulates sarcomeric α-actin, troponin I, myosin light chain 2, the muscle specific M isoform of creatine kinase [11], MyoD and myogenin [12] and these effects have been associated with a specific action of this drug at the transcriptional level. The presence of Dox response elements in the 5′-region of the human Id2 gene [14] suggests a selective effect of the drug on the transcription machinery, as further demonstrated by the inhibition of the transcriptional activity of a crude preparation of RNA pol II [35]. Moreover, it has recently been demonstrated that the presence of the drug in the culture medium can induce transcription from promoters of immediate early genes through an AP-1-independent pathway [36]. Since we have previously shown that the differences in gene expression due to a change in hRPB11 levels are not generalized, as well as the overall transcription rate of housekeeping genes is not changed [10], we suggest that the modulation of hRPB11 observed in Dox resistant cells could be the result of the induction of a specific pathway in response to the Dox stress, and that the presence of low levels of hRPB11 can confer a diminished drug sensitivity to the cell.

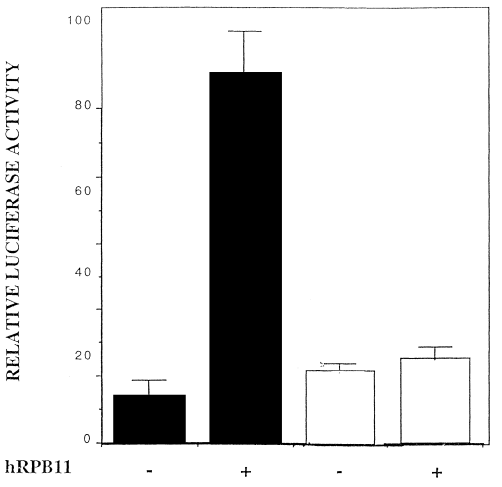

Fig. 3. Relative luciferase activity of the Ecad/Luc and control constructs in the presence or absence of hRPB11 expression vector in LoVoDX cells. Luciferase activities were determined by triplicate transfections of LoVoDx cells with the indicated luciferase construct, a CMV-LacZ control construct, and where indicated, the hRPB11 expression vector. All luciferase activities were normalized for β-galactosidase activity. Filled columns: LoVoDX transfected with Ecad3/Luc. Open columns: LoVoDX transfected with pGL2 control plasmid. Bars: S.D.

In addition, hRPB11 stable transfected cells exhibited a lower proliferative rate and reduced clonogenic ability. LoVoDX cells overexpressing hRPB11 underwent deep alterations in their morphology, displaying increased levels of E-cadherin, the latter due to an increase in transcription most likely the result of an effect of hRPB11 on the E-cadherin promoter. In cancers of epithelial origin, loss of E-cadherin expression has been associated with the loss of differentiated features in tumor cells and/or increased propensity of the cells to invade and metastasize to distant sites [37]. The restoration of E-cadherin expression, following E-cadherin gene transfer, has also been shown to inhibit the invasive and metastatic properties of cells in vitro and animal model systems [38–40].

In agreement with the in vitro data, the evidence of a more differentiated phenotype was proved in vivo, where hRPB11 transfected cells showed reduced tumorigenicity and metastatic ability when inoculated in nude mice.

Thus, taken together, these findings imply that the levels of expression of hRPB11 act as fine regulators of cell differentiation through the transcriptional recruitment of specific genes.

In order to explain how a subunit of the general transcription machinery could achieve such a result, several considerations have to be made. Although hRPB11 is essential for cell viability [41], levels of expression of this protein vary among different tissues with the heart and skeletal muscle expressing the highest [10]. hRPB11 shares a limited amino acid sequence similarity with the α subunit of *Escherichia coli* RNA polymerase and interacts with another human RNA pol II α-like subunit hRPB3. This heterodimer is considered the functional counterpart of bacterial α subunit homodimer [42,43]. The eubacterial α subunit performs at least three critical functions: it serves as the initiator for RNA polymerase assembly, it participates in promoter recognition by sequence-specific protein-DNA interaction, and it is the target for transcriptional regulation by binding to a specific set of transcriptional activator proteins [15,44,45]. This last function is mediated by the C-terminal part of the protein that binds DNA as an isolated entity in a region between −42 and −62 bp, upstream of the transcriptional start site [46]. Although eukaryotic α subunits lack significant homologies with this COOH-terminal of prokaryotic α subunits that plays a role in transcriptional activation, the presence of a highly charged C-terminal flanking sequence in hRPB11 subunit suggests that this domain, in addition to the conserved α-like domain, could be functionally important for interaction with other transcription factors. Moreover, the cnjC gene product, a homologue of the other α-like RNA pol II subunit hRPB3 in *Tetrahymena*, has been found active only during early conjugation, suggesting that it may be involved in the regulation of transcription at this time [47]. Although recently the hRPB11 subunit has been shown

to form a core subassembly subunit of RNA pol II with DNA binding activity [48], to date the function of this protein has remained elusive.

Based on these observations, on the effects of hRPB11 overexpression observed in LoVoDX cells and on the fact that the expression of hRPB11 exhibits a tissue-specific pattern in normal tissues [10], we speculate that hRPB11 may exert its specific activity on gene regulation in a manner not dissimilar from the one recently described for other core promoter elements that mediate preferential transcription of specific promoters [49,50]. We therefore hypothesize that this protein may play a regulatory function in transcription by varying its stoichiometry with other RNA pol II subunits and/or core promoter elements, resembling in certain instances the function of bacterial σ factors [51].

Further investigations of the hRPB11 transfected clones will allow us to determine which genes are specifically modulated by hRPB11 overexpression and will help to define more precisely the function of this eukaryotic RNA pol subunit in the processes of gene transcription and its regulation.

Acknowledgements: We are grateful to A. Mangoni for technical assistance. We thank Dr. E.R. Fearon (University of Michigan, Ann Arbor) for providing us with the E-cadherin promoter constructs. We thank Drs. G. Zupi, C. Passananti, V. Sartorelli, A. Nista and S. Soddu for helpful discussion. R. Bruno is gratefully acknowledged for processing the manuscript for publication. This work was partially supported by Associazione Italiana per la Ricerca sul Cancro, CNR-ACRO.

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